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(54) Title: NUCLEIC ACID ANALYSIS METHODS

(57) Abstract

Methods are provided for detecting the presence of mutant sequences in a subpopulation of gene sequences in a biological sample. These methods are particularly useful for identifying individuals with gene mutations indicative of early colorectal cancer.

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NUCLEIC ACID ANALYSIS METHODS

FIELD OF THE INVENTION

This invention relates generally to methods for using segmented oligonucleotides. Methods of the invention are especially useful for disease diagnosis by detecting the presence of genetic mutations in cellular samples.

BACKGROUND OF THE INVENTION

The knowledge of molecular defects causative of diseases, such as inherited disorders and cancer, is increasing rapidly. Inherited diseases thought to be caused by genetic mutations include sickle cell anemia, α- and β-thalassemias, phenylketonuria, hemophilia, α_1-anti-trypsin deficiency, and cystic fibrosis. Sickle cell anemia, for example, is reported to result from homozygosity resulting from a single base pair substitution in the sixth codon of the β-globin gene. Antonarakis, *New England J. Med., 320*: 153-163 (1989). Mutations in the insulin receptor gene and in the insulin-responsive glucose transporter gene have been detected in insulin-resistant diabetes. Krook *et al., Human Molecular Genetics, 1*: 391-396 (1992).

Cancer has been associated with genetic mutations in a number of oncogenes and tumor suppressor genes. Duffy, *Clin. Chem., 41*: 1410-1413 (1993). For example, point mutations in the *ras* genes have been shown to convert those genes into transforming oncogenes. Bos *et al., Nature, 315*: 726-730. Mutations and the loss of heterozygosity at the p53 tumor suppressor locus have been correlated with various types of cancer. Ridanpaa *et al., Path. Res. Pract., 191*: 399-402 (1995); Hollstein *et al., Science, 253*: 49-53 (1991). In addition, the loss or other mutation of the *apc* and *dcc* tumor suppressor genes has also been associated with tumor development. Blum, *Europ. J. Cancer, 31A*: 1369-1372 (1995). Those mutations can serve as markers for early stages of disease and for predisposition thereto. Early diagnosis is not only

important for successful treatment, but can also lead to prevention or treatment before chronic symptoms occur.

Colorectal cancer is an example of a disease that is highly curable if detected early. With early detection, colon cancer may be effectively treated by, for example, surgical removal of the cancerous tissue. Surgical removal of early-stage colon cancer is usually successful because colon cancer begins in cells of the colonic epithelium and is isolated from the general circulation during its early stages. Thus, detection of early mutations in colorectal cells would greatly increase survival rate. Current methods for detection of colorectal cancer focus on extracellular indicia of the presence of cancer, such as the presence of fecal occult blood or carcinoembryonic antigen circulating in serum. Such extracellular indicia typically occurs only after the cancer has become invasive. At that point, colorectal cancer is very difficult to treat.

Methods have been devised to detect the presence of mutations within disease-associated genes. One such method is to compare the complete nucleotide sequence of a sample genomic region with the corresponding wild-type region. See, *e.g.*, Engelke *et al.*, *Proc. Natl. Acad. Sci, U.S.A., 85*: 544-548 (1988) and Wong *et al.*, *Nature, 330*: 384-386 (1988). However, such methods are costly, time consuming, and require the analysis of multiple clones of the targeted gene for unambiguous detection of low-frequency mutations. As such, it is not practical to use extensive sequencing for large-scale screening of genetic mutations.

A variety of detection methods have been developed which exploit sequence variation in DNA using enzymatic and chemical cleavage techniques. A commonly-used screen for DNA polymorphisms consists of digesting DNA with restriction endonucleases and analyzing the resulting fragments by means of southern blots, as reported by Botstein *et al.*, *Am. J. Hum. Genet.*, 32: 314-331 (1980) and White *et al.*, *Sci. Am.*, 258: 40-48 (1988). Mutations that affect the recognition sequence of the endonuclease will preclude enzymatic cleavage at that site, thereby altering the cleavage pattern of the DNA. Sequences are compared by looking for differences in restriction fragment lengths. A problem with this method (known as restriction fragment length polymorphism mapping or RFLP mapping) is its inability to detect mutations that

do not affect cleavage with a restriction endonuclease. One study reported that only 0.7% of the mutational variants estimated to be present in a 40,000 base pair region of human DNA were detected using RFLP analysis. Jeffreys, *Cell, 18*: 1-18 (1979).

Single base mutations have been detected by differential hybridization techniques using allele-specific oligonucleotide (ASO) probes. Saiki *et al., Proc. Natl. Acad. Sci. USA, 86*: 6230-6234 (1989). Mutations are identified on the basis of the higher thermal stability of the perfectly-matched probes as compared to mismatched probes. Disadvantages of this approach for mutation analysis include: (1) the requirement for optimization of hybridization for each probe, and (2) the nature of the mismatch and the local sequence impose limitations on the degree of discrimination of the probes. In practice, tests based only on parameters of nucleic acid hybridization function poorly when the sequence complexity of the test sample is high (*e.g.*, in a heterogeneous biological sample). This is partly due to the small thermodynamic differences in hybrid stability generated by single nucleotide changes. Therefore, nucleic acid hybridization is generally combined with some other selection or enrichment procedure for analytical and diagnostic purposes.

In enzyme-mediated ligation methods, a mutation is interrogated by two oligonucleotides capable of annealing immediately adjacent to each other on a target DNA or RNA molecule, one oligonucleotide having the 3' end complementary to the point mutation. Adjacent oligonucleotide sequences are only covalently attached when both oligonucleotides are correctly base-paired. Thus, the presence of a point mutation is indicated by the ligation of the two adjacent oligonucleotides. Grossman *et al., Nucleic Acid Research, 22*: 4527-4534 (1994). However, the usefulness of this method for detection is compromised by high backgrounds which arise from tolerance of certain nucleotide mismatches or from non-template directed ligation reactions. Barringer *et al., Gene, 89*: 117-122 (1990).

A number of detection methods have been developed which are based on a template-dependent, primer extension reaction. These methods fall essentially into two categories: (1) methods using primers which span the region to be interrogated for the

mutation, and (2) methods using primers which hybridizes proximally and upstream of the region to be interrogated for the mutation.

In the first category, Caskey and Gibbs [U.S. Patent No. 5,578,458] report a method wherein single base mutations in target nucleic acids are detected by competitive oligonucleotide priming under hybridization conditions that favor the binding of the perfectly-matched primer as compared to one with a mismatch. Vary and Diamond [U.S. Patent No. 4,851,331] described a similar method wherein the 3' terminal nucleotide of the primer corresponds to the variant nucleotide of interest. Since mismatching of the primer and the template at the 3' terminal nucleotide of the primer inhibits elongation, significant differences in the amount of incorporation of a tracer nucleotide result under normal primer extension conditions.

It has long been known that primer-dependent DNA polymerases have, in general, a low replication error rate. This feature is essential for the prevention of genetic mistakes which would have detrimental effects on progeny. Methods in a second category exploit the high fidelity inherent in this enzymological reaction. Detection of mutations is based on primer extension and incorporation of detectable, chain-terminating nucleoside triphosphates. The high fidelity of DNA polymerases ensures specific incorporation of the correct base labeled with a reporter molecule. Such single nucleotide primer-guided extension assays have been used to detect aspartylglucosaminuria, hemophilia B, and cystic fibrosis; and for quantifying point mutations associated with Leber Hereditary Optic Neuropathy (LHON). See. *e.g.*, Kuppuswamy *et al., Proc. Natl. Acad. Sci. USA, 88*: 1143-1147 (1991); Syvanen *et al., Genomics, 8*: 684-692 (1990); Juvonen *et al., Human Genetics, 93*: 16-20 (1994); Ikonen *et al., PCR Meth. Applications, 1*: 234-240 (1992); *Ikonen et al., Proc. Natl. Acad. Sci. USA, 88*: 11222-11226 (1991); Nikiforov *et al., Nucleic Acids Research, 22*: 4167-4175 (1994).

Strategies based on primer extension require considerable optimization to ensure that only the perfectly annealed oligonucleotide functions as a primer for the extension reaction. The advantage conferred by the high fidelity of the polymerases can be compromised by the tolerance of nucleotide mismatches in the hybridization of

the primer to the template. Any "false" priming will be difficult to distinguish from a true positive signal.

The selectivity and stability of the oligonucleotide primer extension assay is determined by the length of the oligonucleotide primer. Under typical reaction conditions, short primers (*i.e.*, less than about a 15-mer) exhibit transient, unstable hybridization and, consequently, do not readily prime the extension reaction. Moreover, in a complex heterogeneous biological sample, short primers exhibit non-specific binding to a wide variety of perfectly-matched complementary sequences. Thus, because of their low stability and high non-specific binding, short primers are not very useful for reliable identification of a mutation at a known location. Therefore, detection methods based on primer extension assays use oligonucleotide primers ranging in length from 15-mer to 25-mer. *See e.g.*, PCT Patent Publications WO 91/13075; WO 92/15712; and WO 96/30545. Lengthening the probe to increase stability, however, has the effect of diminishing selectivity. Due to the small thermodynamic differences in hybrid stability generated by single nucleotide changes, a single base mismatch usually does not affect binding efficiency of longer oligonucleotide primers. This tolerance of nucleotide mismatches in the hybridization of the primer to the template can result in significant levels of non-specific "false" priming in complex heterogeneous biological samples.

Methods in the art reduce the possibility of false priming by decreasing the sequence complexity of the test sample. Thus, genomic DNA is isolated from the biological sample and/or amplified with PCR using primers which flank the region to be interrogated. The primer extension analysis is then conducted on the purified PCR products. *See* PCT Patent Publications WO 91/13075; WO 92/15712; and WO 96/30545. Moreover, since considerable optimization is required to ensure that only the perfectly annealed oligonucleotide functions as a primer for the extension reaction, only limited multiplexing of the primer extension assays is possible. Krook *et al., supra* report that multiplexing can be achieved by using primers of different lengths and by monitoring the wild-type and mutant nucleotide at each mutation site in two separate single nucleotide incorporation reactions. Given that the selectivity and

stability of the oligonucleotide primer extension assay is determined by the length of the oligonucleotide primer, the number of primers that can be tested simultaneously in a given reaction mixture is very limited. Accordingly, these methods are limited in scope due to low throughput.

Therefore, there is a need in the art for simple and efficient detection methods for reliable large-scale screening of a large number of genomic mutations in heterogeneous biological samples. Such methods are provided herein.

SUMMARY OF THE INVENTION

The present invention provides methods for increasing the selectivity of nucleic acid hybridization reactions. Methods of the invention comprises using segmented oligonucleotides in order to simultaneously achieve the hybridization stability characteristic of relatively long probes and the nucleic acid selectivity (*i.e.*, intolerance of mismatches) characteristic of shorter probes. Use of segmented oligonucleotides of the invention allows stable, selective (*i.e.*, perfectly matched) hybridization of oligonucleotide probes for the identification of target nucleic acids, for template-dependent extension of such probes, for cleavage of the nucleic acids or the probes hybridized thereto, and for other nucleic acid reactions that benefit from stable, selective hybridization. Furthermore, methods of the present invention permit the use of multiple segmented oligonucleotides under one set of reaction conditions.

In a preferred embodiment, methods of the invention comprise exposing a sample suspected to contain a target nucleic acid to a short first probe and a longer second probe capable of hybridizing to substantially contiguous portions of the target nucleic acid, thereby to detect the target nucleic acid. The two probes are exposed to sample under conditions that do not favor the hybridization of short first probe in the absence of longer second probe. Factors affecting hybridization are well known in the art and include temperature, ion concentration, pH, probe length, and probe GC content. A first probe, because of its small size, hybridizes numerous places in an average genome. For example, any given 8-mer occurs about 65,000 times in the human genome. However, an 8-mer has a low melting temperature (T_m) and a single

base mismatch greatly exaggerates this instability. A second probe, on the other hand, is larger than the first probe and will have a higher T_m. A 20-mer second probe, for example, typically hybridizes with more stability than an 8-mer. However, because of the small thermodynamic differences in hybrid stability generated by single nucleotide changes, a longer probe will form a stable hybrid but will have a lower selectivity because it will tolerate nucleotide mismatches. Accordingly, under unfavorable hybridization conditions for the first probe (*e.g.*, 10-40°C above first probe T_m), the first probe hybridizes with high selectivity (*i.e.*, hybridizes poorly to sequence with even a single mismatch), but forms unstable hybrids when it hybridizes alone (*i.e.*, not in the presence of a second probe). The second probe will form a stable hybrid but will have a lower selectivity because of its tolerance of mismatches.

Methods of the invention comprise conducting a reaction that would not occur absent contiguous hybridization of the first and second probes. The first and second probes hybridize to substantially contiguous portions of the target. For purposes of the present invention, substantially contiguous portions are those that are close enough together to allow hybridized first and second probes to function as a single probe (*e.g.*, as a primer of nucleic acid extension). Substantially contiguous portions are preferably between zero (*i.e.*, exactly contiguous so there is no space between the portions) nucleotides and about one nucleotide apart. A linker is preferably used where the first and second probes are separated by two or more nucleotides, provided the linker does not interfere with the assay (*e.g.*, nucleic acid extension reaction). Such linkers are known in the art and include, for example, peptide nucleic acids, DNA binding proteins, and ligation. It has now been realized that the adjacent probes bind cooperatively so that the longer, second probe imparts stability on the shorter, first probe. However, the stability imparted by the second probe does not overcome the selectivity (*i.e.*, intolerance of mismatches) of the first probe. Therefore, methods of the invention take advantage of the high selectivity of the short first probe and the hybridization stability imparted by the longer second probe.

By requiring hybridization of the two probes, false positive signals are reduced or eliminated. As such, the use of segmented oligonucleotides eliminates the need for

careful optimization of hybridization conditions for individual probes, as presently required in the art, and permits extensive multiplexing. Several segmented oligonucleotides can be used to probe several target sequences assayed in the same reaction, as long as the hybridization conditions do not permit stable hybridization of short first probes in the absence of the corresponding longer second probes.

Also in a preferred embodiment, methods of the invention comprise improvements in the detection of mutant nucleic acids. A feature of the invention is the recognition that the selectivity of an oligonucleotide primer extension assays is significantly improved with the use of segmented oligonucleotides as primers for template-based extension. In mutation detection methods of the invention, a first (proximal) probe hybridizes adjacent to a nucleic acid suspected to be mutated. Preferably, the first probe comprises between about 5 and about 10 nucleotides. The first probe alone is not a primer for template-based nucleic acid extension because it will not form a stable hybrid under the reaction conditions used in the assay. A second (distal) probe in mutation detection methods of the invention hybridizes upstream of the first probe and to a substantially contiguous region of the target (template). The second probe alone is not a primer of template-based nucleic acid extension because it comprises a 3' non-extendible nucleotide. The second probe is larger than the first probe, and is preferably between about 15 and about 100 nucleotides in length.

According to methods of the invention, template-dependent extension takes place only when a first probe hybridizes next to a second probe. When this happens, the short first probe hybridizes immediately adjacent to the site of the suspected single base mutation. The second probe hybridizes in close proximity to the 5' end of the first probe. The presence of the two probes together increases stability due to cooperative binding effects. Together, the two probes are recognized by polymerase as a primer. This system takes advantage of the high selectivity of a short probe and the hybridization stability imparted by a longer probe in order to generate a primer that hybridizes with the selectivity of a short probe and the stability of a long probe. Accordingly, there is essentially no false priming with segmented primers. Since the tolerance of mismatches by the longer second probe will not generate false signals,

several segmented primers can be assayed in the same reaction, as long as the hybridization conditions do not permit the extension of short first probes in the absence of the corresponding longer second probes.

Thus, in a preferred embodiment, first and second probes are hybridized to substantially contiguous regions of target, wherein the first probe is immediately adjacent and upstream of a site of suspected mutation, for example, a single base mutation. The sample is then exposed to dideoxy nucleic acids that are complements of possible mutations at the suspected site. For example, if the wild-type nucleic acid at a known site is adenine, then dideoxy adenine, dideoxy cytosine, and dideoxy guanine are placed into the sample. Preferably, the dideoxy nucleic acids are labeled. Deoxynucleotides may alternatively be used if the reaction is stopped after the addition of a single nucleotide. Polymerase, either endogenously or exogenously supplied, catalyzes incorporation of a dideoxy base on the first probe. Detection of label indicates that a non-wild-type (*i.e.*, mutant) base has been incorporated, and there is a mutation at the site adjacent the first probe. Alternatively, methods of the invention may be practiced when the wild-type sequence is unknown. In that case, the four common dideoxy nucleotides are differentially labeled. Appearance of more than one label in the assay described above indicates a mutation may exist.

Also, in a preferred embodiment, segmented oligonucleotides, comprising first and second probes, are used to increase the selectivity (*i.e.*, reduce the possibility of false positives) of target nucleic acid detection methods. A sample suspected to contain a target is exposed to first and second probes, wherein the first probe is a shorter, less stable, but selective probe; and the second probe is a longer, more stable, less selective probe. A target is detected by hybridization of the first and second probes to substantially contiguous portions of the target. Preferably, the first and second probes are detectably labeled.

In an alternative preferred embodiment, a segmented oligonucleotide comprises a series of first probes, wherein sufficient stability is only obtained when all members of the segmented oligonucleotide simultaneously hybridize to substantially contiguous portions of a nucleic acid. It has now been realized that, although short probes exhibit

transient, unstable hybridization, adjacent short probes bind cooperatively and with greater stability than each individual probe. Together, a series of adjacently-hybridized first probes will have greater stability than individual probes or a subset of probes in the series. For example, in an extension reaction with a segmented primer comprising a series of three first probes (*i.e.*, three short probes with no terminal nucleotide capable of hybridizing to a substantially contiguous portion of a nucleic acid upstream of the target nucleic acid), the concurrent hybridization of the three probes will generate sufficient cooperative stability for the three probes to prime nucleic acid extension and the short probe immediately adjacent to a suspected mutation will be extended. Thus, segmented probes comprising a series of short first probes offer the high selectivity (*i.e.*, intolerance of mismatches) of short probes and the stability of longer probes.

Methods of the invention are also useful for the detection of heterozygosity at a single-base locus. In such methods, first and second probes, as described above, are constructed such that the first probe hybridizes immediately adjacent to the single base being investigated. The second probe hybridizes to a region that is substantially contiguous with the region to which the first probe hybridizes. Alternatively, the segmented nucleotide can comprise a series of short first probes that can hybridize to a contiguous region immediately adjacent to and upstream of the single base being investigated. The sample is then exposed to the four common dideoxy nucleotides, or to other 3' terminal (*i.e.*, unextendible) nucleotides. A single-base extension reaction is conducted, and the 3' terminal nucleotides incorporated into the first probe are detected. The incorporation of two different, but complementary nucleotides is evidence of heterozygosity at the single base locus. Again, deoxynucleotides may be used if extension is terminated (*e.g.*, by enzyme inactivation) after addition of one nucleotide or if only one labeled nucleotide is present for incorporation during extension.

Further, as described in co-owned, co-pending patent application Serial No. 08/700,583 (Attorney docket No. EXT-001), incorporated by reference herein, methods according to the invention also are useful to detect a loss of heterozygosity at an allele by determination of the amounts of maternal and paternal alleles comprising a

genetic locus that includes at least one single-base polymorphism. A statistically-significant difference in the amounts of each allele is indicative of a mutation in an allelic region encompassing the single-base polymorphism. In this method, a region of an allele comprising a single-base polymorphism is identified. Segmented oligonucleotides are designed to hybridize to corresponding regions on both paternal and maternal alleles immediately 3' to the single base polymorphism. After hybridization, a mixture of at least two of the four common dideoxy nucleotides (or deoxynucleotides if the reaction is stopped after addition of one nucleotide) are added to the sample, each labeled with a different detectable label. A DNA polymerase is also added. Using allelic DNA adjacent the polymorphic nucleotide as a template, the hybridized segmented oligonucleotide is extended by the addition of a single dideoxynucleotide that is the binding partner for the polymorphic nucleotide. After washing to remove unincorporated dideoxynucleotides, the dideoxynucleotides that have been incorporated into the first probe extension are detected by determining the number of bound extended probes bearing each of the two dideoxynucleotides in, for example, a flow cytometer or impedance counter. The presence of an almost equal number of two different labels mean that there is heterozygosity at the polymorphic nucleotide. The presence of a statistically-significant difference between the detected numbers of the two labels means that a deletion of the region encompassing the polymorphic nucleotide has occurred in one of the alleles.

Deoxynucleotides may be used as the detectable single extended base in any of the reactions described above that require single base extension. However, in such methods, the extension reaction must be stopped after addition of the single deoxynucleotide. Such methods may be employed regardless of whether a specific mutation is known (*i.e.*, C→G). Moreover, the extension reaction need not be terminated after the addition of only one deoxynucleotide if only one labeled species of deoxynucleotide is made available in the sample for detection of the single base mutation. This method may actually enhance signal if there is a nucleotide repeat including the interrogated single base position.

Also in a preferred embodiment, methods of the invention may be used to detect target nucleic acid sites for subsequent cleavage of the hybridized (probe/target) nucleic acid. Cleavage is preferably accomplished with an enzyme, for example, a restriction endonuclease.

Further aspects and advantages of the invention are apparent upon consideration of the following detailed description thereof.

DESCRIPTION OF THE DRAWING

Figure 1 is a diagram depicting the use of a segmented primer in a single base extension reaction for the detection of single base polymorphisms. The white bar represents the template, the dark gray bar represents second probe which hybridizes to a region on the template that is substantially contiguous with the first probe (light gray). The site suspected to be a single base mutation is labeled A. The detectable label is marked B.

DETAILED DESCRIPTION OF THE INVENTION

Methods of the invention are useful for a variety of reactions in which substantially contiguous hybridization of two probes is desired. For example, methods of the invention may be used to detect a target nucleic acid sequence using first and second probes as described above. Due to their increased selectivity for target, methods of the invention may be used to detect target nucleic acid that is available in small proportion in a sample and that would normally have to be amplified by, for example, PCR in order to be detected.

A particularly-preferred use of methods of the invention is to detect single-base mutations, especially in a heterogeneous sample, such as stool. Accordingly, methods of the invention are exemplified below by reference to detection of a single-base mutation in an oncogene. It is recognized that the single-base mutation may be part of a larger mutation. Often, however, as with the *ras* oncogenes, a single point mutation may be responsible for activation of the mutation.

Preferred methods of the invention comprise using segmented primers to enhance template-dependent nucleic acid polymerization. Such methods are especially useful for detection of mutations, especially point mutations. Methods of the invention comprise hybridizing two probes adjacent to a site of suspected mutation, wherein neither probe alone is capable of being a primer for template-dependent extension, but wherein adjacent probes are capable of priming extension. In a preferred embodiment, methods of the invention comprise hybridizing to a target nucleic acid a probe having a length from about 5 bases to about 10 bases, wherein the probe hybridizes immediately upstream of a suspected mutation. Methods of the invention further comprise hybridizing a second probe upstream of the first probe, the second probe having a length from about 15 to about 100 nucleotides and having a 3' non-extendible nucleotide. The second probe is substantially contiguous with the first probe. Preferably, substantially contiguous probes are between 0 and about 1 nucleotide apart. A linker is preferably used where the first and second probes are separated by two or more nucleotides, provided the linker does not interfere with the nucleic acid extension reaction. Such linkers are known in the art and include, for example, peptide nucleic acids, DNA binding proteins, and ligation. Finally, methods of the invention comprise conducting an extension reaction to add nucleotides to the segmented primer resulting from co-hybridization of the above-described probes in a template-dependent manner.

Based upon the foregoing explanation, the skilled artisan appreciates that methods of the invention are useful to detect mutations in a subpopulation of a polynucleotides in any biological sample. For example, methods disclosed herein may be used to detect mutations associated with diseases such as cancer. Additionally, methods of the invention may be used to detect a deletion or a base substitution mutation causative of a metabolic error, such as complete or partial loss of enzyme activity. For purposes of exemplification, the following provides details of the use of methods according to the present invention in colon cancer detection. Inventive methods are especially useful in the early detection of a mutation. Accordingly, while exemplified in the following manner, the invention is not so limited and the skilled artisan will appreciate its wide range of applicability upon consideration thereof.

Exemplary Methods for Detection of Colon Cancer or Precancer

I. Sample Preparation

In a preferred embodiment, a sample for analysis according to the invention is selected from stool, urine, sputum, blood, lymphatic fluid, semen, biopsy tissue, cerebrospinal fluid, and pus. In a particularly preferred embodiment, the sample is a cross-section of stool. A preferred method for preparing a cross-section of stool is provided in co-owned, co-pending patent application Serial No. 08/699,678 (Attorney docket No. EXT-002), incorporated by reference herein. As stool passes through the colon, it adheres cells and cellular debris sloughed from colonic epithelial cells. Similarly, cells and cellular debris are sloughed by a colonic polyp (comprising mutated DNA). However, only the portion of stool making contact with the polyp will adhere sloughed cells. It is therefore necessary to obtain at least a cross-section of stool in order to ensure that the stool sample contains a mixture of all sloughed cells, including those sloughed by presumptive cancer cells (*e.g.*, polyps).

After sample preparation, sample is homogenized in an appropriate buffer, such as phosphate buffered saline comprising a salt, such as 20-100 mM NaCl or KCl, and a detergent, such as 1-10% SDS or Triton™, and/or a proteinase, such as proteinase K. The buffer may also contain inhibitors of DNA and RNA degrading enzymes. Double-stranded DNA in the sample is melted (denatured to form single-stranded DNA) by well-known methods *See, e.g.,* Gyllensten *et al., in Recombinant DNA Methodology II*, 565-578 (Wu, ed., 1995), incorporated by reference herein. DNA or RNA may optionally be isolated from the sample according to methods known in the art. See, Smith-Ravin *et al., Gut, 36*: 81-86 (1995), incorporated by reference herein. Once sample is prepared, it is exposed to one or more set of segmented primers according to the invention.

II. Preparation of Segmented Primers

Genomic regions suspected to contain one or more mutations are identified by reference to a nucleotide database, such as GenBank, EMBL, or any other appropriate database or publication, or by sequencing. For cancer detection, genetic mutations in

a number of oncogenes and tumor suppressor genes are known. Duffy, *Clin. Chem.*, *41*: 1410-1413 (1993). Preferred genes for use in mutation detection methods of the invention include one or more oncogenes and/or one or more tumor suppressor genes. Specifically preferred genes include the *ras* oncogenes, *p53*, *dcc*, *apc*, *mcc*, and other genes suspected to be involved in the development of an oncogenic phenotype.

As will be described below, methods of the invention permit the detection of a mutation at a locus in which there is more than one nucleotide to be interrogated. Moreover, methods of the invention may be used to interrogate a locus in which more than one single base mutation is possible. Once regions of interest are identified, at least one segmented primer is prepared to detect the presence of a suspected mutation. A segmented primer comprises at least two oligonucleotide probes, a first probe and a second probe, which are capable hybridizing to substantially contiguous portions of a nucleic acid.

A first probe of the invention preferably has a length of from about 5 to about 10 nucleotides, more preferably between about 6 and about 8 nucleotides, and most preferable about 8 nucleotides. A second probe of the invention has a preferable length of between about 15 and 100 nucleotides, more preferably between about 15 and 30 nucleotides, and most preferably about 20 nucleotides. Further, a second probe is incapable of being a primer for template-dependent nucleic acid synthesis absent a first probe because it has a 3' terminal nucleotide that is non-extendible. Preferred non-extendible 3' terminal nucleotides include dideoxy nucleotides, C3 spacers, a 3' inverted base, biotin, or a modified nucleotide. Although, longer probes have a lower selectivity because of their tolerance of nucleotide mismatches, second probes are non-extendible and will not produce false priming in the absence of the proximal probe.

In an alternative embodiment, a segmented primer comprises a series of first probes, wherein each member of the series has a length of from about 5 to about 10 nucleotides, and most preferable about 6 to about 8 nucleotides. Although the first probes do not have a terminal nucleotide, nucleic acid extension will not occur unless

all members of the series are hybridized to substantially contiguous portions of a nucleic acid.

The oligonucleotide probes of the segmented primer may be natural or synthetic, and may be synthesized enzymatically *in vivo*, enzymatically *in vitro*, or non-enzymatically *in vitro*. Probes for use in methods of the invention are preferably selected from oligodeoxyribonucleotides, oligoribonucleotides, copolymers of deoxyribonucleotides and ribonucleotides, peptide nucleic acids (PNAs), and other functional analogues. Peptide nucleic acids are well-known. See Pluskal, *et al., The FASEB Journal*, Poster #35 (1994). They are synthetic oligoamides comprising repeating amino acid units to which adenine, cytosine, guanine, thymine or uracil are attached. *See* Egholm, *et al., Nature*, 365: 566-568 (1993); Oerum, *et al. Nucl. Acids Res.*, 23: 5332-36 (1993); *Practical PNA: Identifying Point Mutations by PNA Directed PCR Clamping*, PerSeptive Biosystems Vol. 1, Issue 1 (1995). Peptide nucleic acid synthons and oligomers are commercially available form PerSeptive Biosystems, Inc., Framingham, MA. *See, e.g.*, PCT publications EP 92/01219, EP 92/01220, US92/10921. In many applications, PNA probes are preferred to nucleic acid probes because, unlike nucleic acid/nucleic acid duplexes, which are destabilized under conditions of low salt, PNA/nucleic acid duplexes are formed and remain stable under conditions of very low salt. Additionally, because PNA/DNA complexes have a higher thermal melting point than the analogous nucleic acid/nucleic acid complexes, use of PNA probes can improve the reproducibility of blotting assays.

For exemplification, probes designed to detect mutations in the *K-ras* gene are provided below. According to methods of the invention, probes complementary to either portions of the coding strand or to portions of the non-coding strand may be used. For illustration, probes useful for detection of mutations in the coding strand are provided below. Mutations in *K-ras* frequently occur in the codon for amino acid 12 of the expressed protein. Several of the possible probes for detection of mutations at each of the three positions in codon 12 are shown below.

The wild-type codon 12 of the *K-ras* gene and its upstream nucleotides are:

wild-type template 3'- TATTTGAACACCATCAACCTCGACCA-5' (SEQ ID NO:1)

The three nucleotides encoding amino acid 12 are underlined. First probes and second probes capable of interrogating the three nucleotides coding for amino acid 12 of the *K-ras* gene are provided below. First probe A is a first probe as described generally above, and has a sequence complementary to the nucleotides immediately upstream of the first base in codon 12 (*i.e.*, immediately adjacent to the cytosine at codon position 1). Second probe A is a second probe as generally described above. It is complementary to a sequence that is substantially contiguous (here, exactly contiguous) with the sequence to which the first probe A is complementary. The bolded nucleotide in each of the second probes shown below is the nonextendible 3' terminal nucleotide. Hybridization of first and second probes suitable for detection of a mutation in the first base of K-ras codon 12 are shown below:

second probe A	5'-ATAAACTTGTGGTAG	(SEQ ID NO: 2)
first probe A	TTGGAGCT	(SEQ ID NO: 3)
wild-type template	3'-TATTTGAACACCATCAACCTCGACCA-5'	(SEQ ID NO: 1)

Detection of a mutation in the second base in codon 12 may be performed by using the same second probe as above (second probe A), and a first probe, identified as first probe B below, that is complementary to a sequence terminating immediately adjacent (3') to the second base of codon 12. Hybridization of probes suitable for detection of a mutation in the second base of codon 12 are shown below:

second probe A	5'-ATAAACTTGTGGTAG	(SEQ ID NO: 2)
first probe B	TGGAGCTG	(SEQ ID NO: 4)
wild-type template	3'-TATTTGAACACCATCAACCTCGACCA-5'	(SEQ ID NO: 1)

Detection of a mutation at the third position in codon 12 is accomplished using the same second probe as above, and first probe C, which abuts the third base of codon 12. Hybridization of probes suitable for detection of a mutation in the third base of codon 12 are shown below

second probe A	5'-ATAAACTTGTGGTAG	(SEQ ID NO: 2)
first probe C	GGAGCTGG	(SEQ ID NO: 6)
wild-type template	3'-TATTTGAACACCATCAACCTCGACCA-5'	(SEQ ID NO: 1)

In methods for detection of mutations at the second and third nucleotides of codon 12 described above, the second probe is 1 and 2 nucleotides, respectively, upstream of the region to which the first probe hybridizes. Alternatively, second probes

for detection of the second and third nucleotides of codon 12 may directly abut (*i.e.*, be exactly contiguous with) their respective first probes. For example, an alternative second probe for detection of a mutation in the third base of codon 12 in K-ras is:

5'-ATAAACTTGTGGTAGTT (SEQ ID NO: 5)

The detection of mutations can also be accomplished with a segmented primer comprising a series of at least three first probes. A series of first probes suitable for detection of a mutation in the third base of codon 12 is shown below:

first probe X	5'-ATAAACTT	(SEQ ID NO: 7)
first probe Y	TGGTAGTT	(SEQ ID NO: 8)
first probe Z	GGAGCTGG	(SEQ ID NO: 6)
wild-type template	3'-TATTTGAACACCATCAACCTCGACCA-5'	(SEQ ID NO: 1)

III. Single base primer extension assays

First and second probes are exposed to sample under hybridization conditions that do not favor the hybridization of the short first probe in the absence of the longer second probe. Factors affecting hybridization are well known in the art and include raising the temperature, lowering the salt concentration, or raising the pH of the hybridization solution. Under unfavorable hybridization conditions (*e.g.*, at a temperature 30-40 °C above first probe T_m), first probe forms an unstable hybrid when hybridized alone (*i.e.*, not in the presence of a second probe) and will not prime the extension reaction. The longer, second probe, having a higher T_m, will form a stable hybrid with the template and, when hybridized to substantially contiguous portions of the nucleic acid, the second probe will impart stability to the shorter first probe, thereby forming a contiguous primer.

Following the hybridization, the sample may optionally be washed to remove unhybridized probes. In a preferred embodiment, a modification of the dideoxy chain termination method as reported in Sanger, *Proc. Nat'l Acad. Sci. (USA), 74*: 5463-5467 (1977), incorporated by reference herein, is then used to detect the presence of a mutation. The method involves using at least one of the four common 2', 3'-dideoxy nucleoside triphosphates (ddATP, ddCTP, ddGTP, and ddTTP). A detectable detection moiety can be attached to the dideoxy nucleoside triphosphates (ddNTPs) according to methods known in the art. A DNA polymerase, such as Sequenase™ (Perkin-Elmer), is

also added to the sample mixture. Using the substantially contiguous first and second probes as a primer, the polymerase adds one ddNTP to the 3' end of the first probe, the incorporated ddNTP being complementary to the nucleotide that exists at the single-base polymorphic site. Because the ddNTPs have no 3' hydroxyl, further elongation of the hybridized probe will not occur. Chain termination will also result where there is no available complementary ddNTP (or deoxynucleoside triphosphates) in the extension mixture. After completion of the single base extension reaction, extension products are isolated and detected.

Also in a preferred embodiment, labeled deoxynucleotides may be used for detection if either the extension reaction is stopped after addition of only one nucleotide or if only one labeled nucleotide, corresponding to the complement of the expected mutation, is exposed to the sample.

In the simplest embodiment of the invention, the nucleoside triphosphate mixture contains just the labeled ddNTP or dNTP complementary to the known mutation. For example, to interrogate a sample for a C→A mutation in the first nucleotide of codon 12 of the *K-ras* gene, second probe A and first probe A are exposed to an extension reaction mixture containing labeled ddTTP or dTTP. The incorporation of a labeled ddTTP or dTTP in first probe A indicates the presence of a C→A mutation in the first nucleotide of codon 12 of the *K-ras* gene in the sample tested. First probe A co-hybridized with second probe A to a wild-type template will not be extended or, alternatively, will be extended with unlabeled ddGTP or dGTP if available in the reaction mixture.

Given the large number of mutations that have been associated with colorectal cancer, a detection method for this disease preferably screens a sample for the presence of a large number of mutations simultaneously in the same reaction (*e.g.*, apc, K-ras, p53, dcc, MSH2, and DRA). As described above, only very limited multiplexing is possible with detection methods of the prior art. Since methods of the present invention eliminate false positive signals resulting from the tolerance of mismatches of the longer second probes, the use of segmented oligonucleotide avoids the need for optimization of hybridization conditions for individual probes and permits

extensive multiplexing. Several segmented primers can be assayed in the same reaction, as long as the hybridization conditions do not permit stable hybridization of short first probes in the absence of the corresponding longer second probes.

In a preferred embodiment, the primer extension reactions are conducted in four separate reaction mixtures, each having an aliquot of the biological sample, a polymerase, and the three labeled complementary non-wild-type ddNTPs (or dNTPs). Optionally, the reaction mixtures may also contain the unlabeled complementary wild-type ddNTP (or dNTP). The segmented primers are multiplexed according to the wild-type template. In the present examplification, the first two nucleotides coding for amino acid 12 of the *K-ras* gene are cysteines. Accordingly, second probe A and first probes A and B are added to a reaction mixture containing labeled ddATP (or dATP), ddTTP (or dTTP), and ddCTP (or dCTP). Second probe C and first probe C are added to a reaction mixture containing labeled ddATP (or dATP), ddCTP (or dCTP), and ddGTP (or dGTP). Any incorporation of a labeled ddNTP in a first probe indicates the presence of a mutation in codon 12 of the *K-ras* gene in the sample. This embodiment is especially useful for the interrogation of loci that have several possible mutations, such as codon 12 of *K-ras*.

In an alternative preferred embodiment, the primer extension reactions are conducted in four separate reaction mixtures, each containing only one labeled complementary non-wild-type ddNTP or dNTP and, optionally, the other three unlabeled ddNTPs or dNTPs. Segmented primers can be thus be exposed only to the labeled ddNTP or dNTP complementary to the known mutant nucleotide or, alternatively, to all three non-wild-type labeled ddNTPs or dNTPs. In the *K-ras* example provided above, if the first nucleotide of *K-ras* codon 12 is interrogated for a known C→G mutation, first probe A and second probe A are added to only one reaction mixture, the reaction mixture containing labeled ddCTP (or dCTP). Optionally, methods of the invention may be practiced as described above using labeled deoxynucleotides.

However, since several mutations have been identified at codon 12 of the *K-ras* gene, the probes are exposed to all non-wild-type labeled ddNTPs or dNTPs. Thus, second probe A and first probes A and B are added to the three reaction mixtures.

containing labeled ddATP (or dATP), ddTTP (or dTTP), or ddCTP (or dCTP). Second probe C and first probe C are added to the three reaction mixtures containing one of labeled ddATP (or dATP), ddCTP (or dCTP), and ddGTP (or dGTP). Again, the extension of a first probe with a labeled terminal nucleotide indicates the presence of a mutation in codon 12 of the *K-ras* gene in the biological sample tested.

IV. Methods for Detection of Labeled Nucleotides

Labeled ddNTPs or dNTPs preferably comprise a "detection moiety" which facilitates detection of the short probes that have been extended with a labeled terminal nucleotide. Detection moieties are selected from the group consisting of fluorescent, luminescent or radioactive labels, enzymes, haptens, and other chemical tags such as biotin which allow for easy detection of labeled extension products. Fluorescent labels such as the dansyl group, fluorescein and substituted fluorescein derivatives, acridine derivatives, coumarin derivatives, pthalocyanines, tetramethylrhodamine, Texas Red®, 9-(carboxyethyl)-3-hydroxy-6-oxo-6H-xanthenes, DABCYL® and BODIPY® (Molecular Probes, Eugene, OR), for example, are particularly advantageous for the methods described herein. Such labels are routinely used with automated instrumentation for simultaneous high throughput analysis of multiple samples.

Following the extension reaction, first probes (comprising a portion of a segmented primer) that have been extended with a labeled terminal nucleotide are separated from unincorporated labeled ddNTPs or dNTPs. Such separation is accomplished by methods known in the art.

In a preferred embodiment, first probes comprise a "separation moiety." Such separation moiety is, for example, hapten, biotin, or digoxigenin. The separation moiety in first probes does not interfere with the first probe's ability to hybridize with template and be extended. In an alternative embodiment, the labeled ddNTPs comprise a separation moiety. In yet another alternative embodiment, both the first probes and the labeled ddNTPs comprise a separation moiety. Following the extension reaction, a high molecular weight molecule having affinity for the separation moiety (*e.g.*, avidin, streptavidin, or anti-digoxigenin) is added to the reaction mixture under conditions which permit the high molecular weight molecule to bind to the separation

moiety. The reaction components are then separated on the basis of molecular weight using techniques known in the art such as gel electrophoresis, chromatography, or mass spectroscopy. *See*, Ausubel *et al.*, *Short Protocols in Molecular Biology, 3rd ed.* (John Wiley & Sons, Inc., 1995); Wu *Recombinant DNA Methodology II*, (Academic Press, 1995).

In an alternative preferred embodiment, first probes are immobilized to a solid support after extension as described above. The solid support is selected from the group consisting of glass, plastic, and paper. The support is fashioned as a column, bead, dipstick, test tube. In a preferred embodiment, the support is a microtiter dish, having a multiplicity of wells. The conventional 96-well microtiter dishes used in diagnostic laboratories and in tissue culture are a preferred support. The use of such a support allows the simultaneous determination of a large number of samples and controls, and thus facilitates the analysis. Moreover, automated systems can be used to provide reagents to such microtiter dishes.

Any of a variety of methods known in the art may be used to immobilize short probes to a solid support. A commonly used method consists of the non-covalent coating of the solid support with streptavidin or avidin and the immobilization of biotinylated oligonucleotide probe. In this case, first probes comprise at least one biotinylated nucleotide.

Following immobilization of first probes on the support, the support is washed to remove any unbound probe. The support is then tested for the presence of short probes that have been extended with a labeled terminal nucleotide by, for example, spectrophotometric methods.

In an alternative embodiment, the first probes are immobilized on the solid support prior to the extension reaction. The immobilized first probes are then exposed to the sample, the second probes, a polymerase, and labeled ddNTPs or dNTPs. Following the extension reaction, the support is washed to remove the unincorporated labeled ddNTPs and the other reaction mixture components and the support is for the presence of short probes that have been extended with a labeled terminal nucleotide.

The foregoing exemplifies practice of the invention in the context of multiple mutation detection using segmented primers. As disclosed herein, numerous additional aspects and advantages of the invention are apparent upon consideration of the disclosure and the specific exemplification. Accordingly, the invention is limited only by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SHUBER, Anthony P

(ii) TITLE OF INVENTION: IMPROVED NUCLEIC ACID ANALYSIS METHODS

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
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(A) APPLICATION NUMBER:
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MEYERS, Thomas C
(B) REGISTRATION NUMBER: 36,989
(C) REFERENCE/DOCKET NUMBER: EXT-004

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACCAGCTCCA ACTACCACAA GTTTAT 26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATAAACTTGT GGTAG 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTGGAGCT 8

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGAGCTG 8

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATAAACTTGT GGTAGTT 17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGCTGG 8

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATAAACTT 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGTAGTT 8

CLAIMS

What is claimed is:

1. A nucleic acid analysis method, comprising the steps of:

exposing a sample to first and second oligonucleotide probes that hybridize to substantially contiguous regions of a target nucleic acid;

said first probe having low hybridization stability and higher nucleic acid target selectivity and said second probe having a sequence longer than said first probe and having a greater hybridization stability than said first probe;

thereby to produce a substantially contiguous hybridization pair having greater selectivity and higher hybridization stability than either said first or second probe alone; and

conducting a reaction that will not occur absent formation of said substantially contiguous hybridization pair.

2. A nucleic acid analysis method, comprising the steps of:

exposing a sample to first and second oligonucleotide probes that hybridize to substantially contiguous regions of a target nucleic acid;

said first probe having low hybridization stability and higher nucleic acid target selectivity and said second probe having a sequence longer than said first probe and having a greater hybridization stability than said first probe;

thereby to produce a substantially contiguous hybridization pair having greater selectivity and higher hybridization stability than either said first or second probe alone; and

detecting a portion of the target to which said substantially contiguous hybridization pair hybridizes.

3. The method of claim 1, wherein said reaction is a template-based nucleic acid extension reaction.

4. The method of claim 1, wherein said reaction is catalyzed by a ligase.

5. The method of claim 1, wherein said reaction is a reaction that cleaves said hybridization pair.

6. The method of claim 5, wherein said reaction is catalyzed by a restriction endonuclease.

7. The method of claim 1 wherein said first probe is between about 5 nucleotides and about 10 nucleotides in length.

8. The method of claim 1, wherein said second probe is between about 15 nucleotides and about 50 nucleotides in length.

9. The method of claim 1, wherein said sample is selected from the group consisting of stool, pus, blood, sputum, semen, urine, and lymph.

10. The method of claim 1, wherein said second probe comprises a non-extendible 3' nucleic acid.

11. The method of claim 1, wherein said first probe and said second probe are exactly contiguous with each other.

12. The method of claim 1, wherein said second probe is a peptide nucleic acid.

13. A method for improving the selectivity of a nucleic acid hybridization comprising the steps of:

exposing a sample suspected to contain a target nucleic acid to a first oligonucleotide probe comprising from about 5 to 10 nucleotides, and only being capable of hybridizing with a portion of the target in the presence of a second oligonucleotide probe; and

exposing the sample to said second oligonucleotide probe comprising from about 15 to about 50 nucleotides, wherein the second oligonucleotide probe is capable of hybridizing to a region of the target that is substantially contiguous with the portion of the target to which the first oligonucleotide probe hybridizes.

14. A method for detecting a mutation in a nucleic acid, comprising the steps of:

exposing a biological sample to a plurality of oligonucleotide probes, wherein no member of said plurality is capable of being a primer for nucleic acid polymerization unless at least two of said members hybridize to substantially contiguous portions of said nucleic acid immediately upstream of said mutation, thereby to form a contiguous primer;

conducting a single-base extension reaction in said sample, thereby to add a single terminal nucleotide to said contiguous primer; and

detecting a mutation in said nucleic acid as the addition to said contiguous primer of a single terminal nucleotide non-complementary with the wild-type nucleotide.

15. The method according to claim 14, wherein said non-wild-type complementary terminal nucleotide is haptenenated and the detection of said first probe extended with said non-wild-type complementary terminal nucleotide comprises the step of:

exposing said sample to a hapten-specific detectably-labeled high molecular weight molecule; and

separating said non-wild-type extended proximal probe on a molecular weight basis.

16. The method according to claim 1, wherein a plurality of segmented primers are exposed simultaneously to sample.

17. A method for detecting heterozygosity at a single-nucleotide polymorphic locus in a biological sample, comprising the steps of:

exposing a biological sample to a first probe capable of selectively hybridizing to a first portion of a nucleic acid immediately upstream of said locus, wherein said first probe is not a primer for nucleic acid polymerization absent addition to the sample of a second probe;

exposing the sample to a second probe capable of hybridizing to a second portion of the nucleic acid substantially contiguous with and upstream of said first portion, wherein said second probe comprises a non-extendible terminal nucleotide;

exposing the sample to a plurality of different labeled terminal nucleotides;

conducting a single-base extension reaction, thereby to extend said first probe by an additional 3' nucleotide; and;

determining which of said terminal nucleotides are incorporated into said first probe; and

detecting heterozygosity at the single-nucleotide polymorphic site as the detection of two different dideoxy nucleotides having been incorporated into said first probe.

18. The method according to claim 15, wherein said biological sample is selected from the group consisting of pus, blood, urine, sputum, semen, saliva, cerebrospinal fluid, biopsy tissue, and stool.

19. The method according to claim 15, wherein said polymorphic locus is identified from a database of nucleotide sequences.

20. A method for detecting a mutation in a nucleic acid, comprising the steps of:

exposing a biological sample to a series of oligonucleotide probes, wherein no member of said series is capable of being a primer for nucleic acid polymerization unless every member of said series hybridize simultaneously to substantially contiguous portions of said nucleic acid immediately upstream of said mutation, thereby to form a contiguous primer;

conducting a single-base extension reaction in said sample, thereby to add a single terminal nucleotide to said contiguous primer; and

detecting a mutation in said nucleic acid as the addition to said contiguous primer of a single terminal nucleotide non-complementary with the wild-type nucleotide.

21. The method according to claim 20, wherein said series of first oligonucleotide probes comprise three 8-mer members capable of hybridizing to substantially contiguous regions of a target nucleic acid.

22. The method according to claim 20, wherein said series of first oligonucleotide probes comprise four 6-mer members capable of hybridizing to substantially contiguous regions of a target nucleic acid.

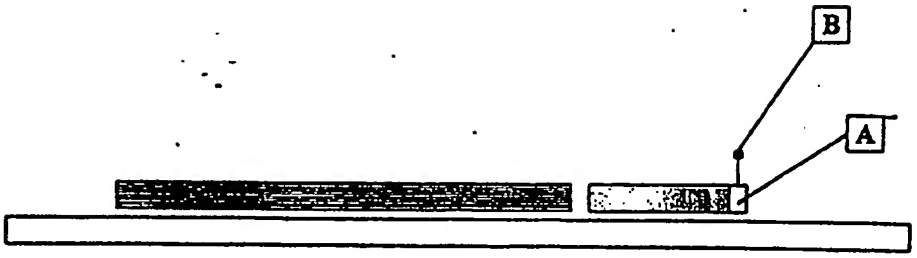

FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/04003

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 185 494 A (APPLIED BIOSYSTEMS) 25 June 1986 see page 5, line 29 - page 7, line 33; claims 13,14,25,26; example 1	1,2,4-9, 11,13 3,14-20
Y	WO 92 15712 A (MOLECULAR TOOL INC) 17 September 1992 cited in the application see page 10 - page 13	3,14-20
X A	WO 93 06240 A (CYTOCELL LTD) 1 April 1993 see page 4, column 2 - page 6, column 1	13 1,2,4,7, 8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

' Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 1998	18/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/04003

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